REMARKS

Receipt of the Office Action mailed September 6, 2007 is hereby acknowledged. With the attached Petition for a One-Month Extension of Time, this response is timely as it is being filed on January 7, 2008 and January 6, 2008 was a Sunday. Reconsideration of the rejections and allowance of this application, as amended, are respectfully requested.

Amendments

Claim 1 has been cancelled and rewritten as new claim 21 in more conventional U.S. form, including a further specification of the hesperidin derivatives which may be used as part of the claimed methods. New claim 21 is supported generally in the specification (for example p. 9, lines 15-33, and p. 19, line 33), and by cancelled claim 1. Claims 2-11 and 13-20 have been amended to depend from new claim 21 and to conform with more conventional U.S. claim language. Claim 12 was cancelled. New claim 21 depends from claim 8 and recites a "preferably" clause which has been deleted from claim 8.

No new matter has been added through the foregoing amendments, and their entry is respectfully requested.

Rejection Under 35 U.S.C. § 101

Claims 1-11 and 13-20 were rejected under 35 U.S.C. § 101 on the grounds that the claims were mere "use" claims. The claims have been rewritten as "method of treatment" claims, obviating this rejection. Reconsideration and withdrawal of the § 101 rejection is requested.

Rejections Under 35 U.S.C. § 112

Claims 4, 5, 7, 15, 16, and 18 have been rejected under 35 U.S.C. § 112, first paragraph and not being enabled for the full scope of the invention. According to the Examiner, the specification is not enabling for the full scope of the invention because, apparently, the Examiner believes it would be "impossible to totally prevent" the disorders identified in the claims at issue.

Applicants strongly traverse this rejection.

First, contrary to the Examiner's argument, the specification does contain working examples showing the preventative activity of the presently claimed method. However, Examples 1 and 3 in the specification describe the effect of the administration of hesperidin on bone metabolism in oophorectonised (also known as ovariectomized or OVX) rats. The reported results demonstrate the efficacy of a method in which hesperidin is administered to subjects in need thereof to stimulate bone formation and inhibit bone resorption. The examples show that the claimed method can prevent bone loss because, as explained below, effective bone loss only occurs in OVX rats after a certain period of time.

The attached article by Shen, et al., Bone, Vol. 20 (No. 1): 55-61 (1997) ("Shen") discloses an academic study of the time course of femoral neck osteopenia in OVX rats. In the "Discussion" section of the paper, the authors wrote: "cancellous bone loss in the proximal tibia of OVX rats is statistically significant as early as 14 days post ovariectomy." (p. 59). This shows that bone loss in OVX rats only after at least 14 days post-ovariectomy. It flows from this teaching that under the conditions of the experiments in Examples 1 and 3 of the present application, the claimed method was effective at least during a period of time of 14 days post-ovariectomy, during which time no bone loss occurred. Thus, the claimed method was able to prevent bone loss. The working examples show not only therapeutic effectiveness, but also prophylactic effect.

Moreover, contrary to the Examiner's statement, the claims do not recite or require total prevention, but rather induction of a preventative effect that would at least postpone or reduce the occurrence of the bone disorder.[1] In this regard, numerous U.S. patents have been granted[2] which recite the "prevention" of bone disorders – such as osteoporosis – which shows that the term "prevention" may be used even if a claimed method does not totally prevent the bone disorder. While applicants recognize that the use a term in other granted patents does not necessarily make its use acceptable in a particular application, in this case it seems clear that claims for methods of "preventing" bone disorders are generally allowable as complying with the requirement of § 112, first paragraph.

In view of the foregoing, applicants respectfully request reconsideration and withdrawal of the enablement rejection.

The Examiner also rejected claims 1-11 and 13-20 under 35 U.S.C. § 112, second paragraph on the grounds that the term " a derivative" as used in claim 1 was indefinite, and the recitation of the claims as "use" claims also rendered them indefinite. Applicants have obviated these rejections by the cancellation of claim 1 and the introduction of new claim 21.

In accordance with the foregoing, applicants submit that the claims are in full compliance with the requirements of § 112.

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[1] For instance the claims at issue – claims 4, 5, 7, 15, 16, and 18 – recite "a method of stimulating bone formation and/or inhibiting bone resorption" by administering a composition, where the composition "is designed to prevent" various ailments.

[2] See, for example, U.S Patent Nos. 7,158,835 (claim 1), 6,846,496 (claim 1), 6,756,401 (claim 3), 6,613,758 (claim 1), 6,511,685 (claim 18), 6,482,809 (claim 1), 6,441,041 (claim 1), 6,440,446 (claim 1), 6,417,224 (claim 1), 6,372,728 (claim 25), 6,340,703 (claim 1), 6,319,255 (claim 1), 6,150,346 (claim 6), 6,133,230 (claim 1), 6,077,872 (claim 1), 6,043,026 (claim 16), 5,985,905 (claim 8), 5,836,997 (claim 1), and 5,782,875 (claim 1). Undoubtedly, numerous other examples could also be found.

Rejections under 35 U.S.C. § 102(b)

The Examiner has rejected claims 1-9 and 11-20 under 35 U.S.C. § 102(b) as being anticipated by Wenzel, et al., EP 1127572A2 ("Wenzel"). According to the Examiner, Wenzel teaches that "compositions of flavone-type compounds of formula I are useful in the treatment of [COX-2] and [NFχB] mediated diseases." Furthermore, according to the Examiner, who cites two different journal articles, COX-2 and NFχB mediated diseases include post-menopausal osteoporosis and other diseases. Based on this, the Examiner concludes: "the limitations of claim 1 are met." Applicants traverse this rejection.

Wenzel relates to the use of flavones for treating COX-2 and NFχB, particularly for treating arthritis and Alzheimer's disease (see ¶0001). Wenzel prescribes the use of flavone compounds for inhibiting the biosynthesis of COX-2 and of NFχB, i.e. as inhibitors of the prostaglandin synthesis. (¶¶ 0001-0005). Numerous compounds that can be used, according to Wenzel, as COX-2 and NFχB inhibitors are shown in Tables 1 to 4 (see pages 3 to 5). According to Wenzel, the diseases linked to COX-2 consist of diseases linked to inflammation, mitogenesis, and ovulation (¶ 0002). Paragraph 0023 specifies a general and theoretical list of diseases which might "potentially" be treated by Wenzel's compounds, including osteoporosis among 16 categories of diseases. The only experimental results disclosed by Wenzel are disclosed in Example 3, which shows that an unidentified flavone compound induces an inhibition of the expression level of the messenger RNA's corresponding to the transcription product of the genes encoding COX-2 and NFχB.

Thus, Wenzel discloses experimental results relating to the inhibition of the expression of the genes and coding for COX-2 and NFχB by an unidentified flavone. Based on this experimental result, Wenzel asserts that flavone compounds of formulae I and II generally inhibit

COX-2 and NFχB and thus are inhibitors of prostaglandin biosynthesis. Therefore, these flavone compounds might potentially be useful, according to Wenzel, for treating diseases linked to prostaglandin and NFχB synthesis, including osteoporosis, a disease linked to bone metabolism.

Wenzel does not anticipate claim 1 – or claim 21 – of the presently claimed invention.

First, there is no evidence that Wenzel enables the use of hesperidin or it derivatives to stimulate bone formation or inhibit bone resorption.

Second, the presently claimed method of stimulating bone formation and/or inhibiting bone resorption by administration of hesperidin or certain hesperidin derivatives is not disclosed by Wenzel. Wenzel generally teaches an inhibition of COX-2 and/or NFχB biosynthesis, not the effect of hesperidin and certain hesperidin derivatives on bone metabolism. Wenzel's disclosure of an alleged effect of flavones generally on the expression of COX-2 and/or NFχB would not teach a person of ordinary skill in the art that hesperidin or certain of its derivatives possess other biological properties, in particular the property of stimulating bone formation and/or inhibiting bone resorption.

As noted above, the only experimental results reported in Wenzel show that an unidentified flavone compound inhibits the expression of the COX-2 and NFχB genes. Although Wenzel does mention hesperidin (as one of 31 different compounds) as one of the compounds falling within the two classes of compounds it is concerned with, and does mention osteoporosis as disease which might be treatable using the disclosed compounds (among 16 total diseases), nowhere does Wenzel specifically or inferentially state or suggest that hesperidin or certain of its derivatives could be administered to a patient to stimulate bone formation or inhibit bone resorption. In fact,

Wenzel does not even state that its compounds could be used to treat osteoporosis (among 16 other diseases), only that they "may potentially be useful in the treatment" of such diseases.

Based on the foregoing, it is apparent that Wenzel does not anticipate applicants' claim 1 (or claim 21). Because the only independent claim is not anticipated, none of the dependent claims can be anticipated either. Therefore, applicants respectfully request withdrawal of the § 102(b) rejection over Wenzel.

Claims 1-7, 9-10, and 12-19 have been rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Kise, et al., JP 2001114675A ("Kise"). The Examiner claims that Kise teaches a vitamin composition containing vitamin K and flavonoids, including hesperidin, and that Kise further teaches that "compositions of vitamin K, vitamin D3, estrogen, isoflavone, etc. are known to prevent and treat osteoporosis." Applicants respectfully traverse this rejection. First, the passage specifically cited by the Examiner and quoted above does not identify hesperidin has being used to treat osteoporosis.

Kise discloses the use of vitamin K in combination with a soybean hypcotyls extract and various other compounds for the treatment of osteoporosis. Hesperidin is only disclosed as being useful to stabilize the unstable vitamin K (¶¶ 0012-0015). Kise simply does not disclose a method of stimulating bone formation and/or inhibiting bone resorption by the administration of hesperidin. Accordingly, the rejection under § 102(b) must be reconsidered and withdrawn.

Rejection Under 35 U.S.C. § 103(a)

The Examiner has rejected claim 9 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Wenzel in view of Barnes, et al., U.S. Patent No. 5,506,211 ("Barnes").

Applicants traverse this rejection.

Barnes discloses the use of a specific isoflavone – genistein – in the treatment of osteoporosis. However, genistein is a completely different compound from hesperidin. Genistein falls within the class of isoflavones while hesperidin is a flavone. As noted above, Wenzel does not teach (or suggest) to one of skill in the art a method of stimulating bone formation or inhibiting bone resorption by the administration of hesperidin. Therefore, that Barnes teaches the use of a completely different compound in foodstuffs to treat osteoporosis does nothing to make claim 9 obvious. Accordingly, applicants respectfully request the withdrawal of the rejection over Wenzel in view of Barnes.

Conclusion

In view of the foregoing, this application is now in condition for allowance. If the examiner believes that an interview might expedite prosecution, the examiner is invited to contact the undersigned.

Respectfully submitted,

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Time Course of Femoral Neck Osteopenia in Ovariectomized Rats

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To characterize osteopenic changes in the femoral neck of ovariectomized (ovx) rats, female Sprague–Dawley rats were ovx or sham operated upon at 3 months of age and killed at various times from 0 to 360 days postsurgery. Quantitative bone histomorphometry was performed on undecalcified longitudinal sections of the proximal femur from each rat. This skeletal site was found to be slowly growing, as its rate of longitudinal bone growth in 3-month-old baseline control rats (5 μm/day) was nearly a factor of 10 less than that of a more commonly used sample site, the proximal tibia. In control rats, cancellous bone volume and cortical bone width of the femoral neck remained relatively constant, but cancellous mineral apposition rate declined with age during the course of the study. In contrast, cancellous bone volume in ovx rats was significantly decreased to 75%–82% of control level at 30–90 days and further decreased to 50%–56% of control level at later times postovariectomy. Indices of cancellous bone turnover such as osteoclast and osteoblast surfaces and bone formation rate were markedly increased in ovx rats at 30 days, declined toward control levels by 90 days, then increased moderately at 180–360 days. In comparison to control rats, a slight decrease in cortical width of the femoral neck was observed in ovx rats at 180 days and reached statistical significance at 360 days postovariectomy. Endocortical bone formation rate was increased significantly in ovx rats compared with control rats at most time points. The results indicate that both cancellous and cortical osteopenia associated with high bone turnover occur in the femoral neck of ovx rats. Cancellous bone loss at this skeletal site is statistically significant as early as 30 days postovariectomy, but remains relatively moderate for the first 90 days before becoming more pronounced at later times after ovariectomy. In contrast, cortical osteopenia was not observed in the femoral neck of ovx rats until 1 year postovariectomy. This histomorphometric characterization of osteopenic changes in the femoral neck of ovx rats may serve as a basis for use of this slowly growing sample site in preclinical studies of the prevention and treatment of bone loss in the estrogen-depleted skeleton. (*Bone 20:55–61; 1997*) *© 1997 by Elsevier Science Inc.*

Key Words: Ovariectomy; Femoral neck; Osteopenia; Bone turnover; Bone histomorphometry.

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Introduction

Osteoporotic hip fracture is a major cause of morbidity and mortality in the elderly.[5,13] Therefore, the proximal femur of the ovx rat, an animal model for postmenopausal bone loss,[8,29] may be a more clinically relevant sample site than other skeletal sites in the appendicular skeleton (i.e., proximal tibia) for preclinical testing of new therapeutic agents for the prevention and treatment of osteopenia. For this reason, the femoral neck has been used in some studies to evaluate by biomechanical techniques bone quality in ovx rats treated with vehicle or test agents.[1,9,23] The results of these studies indicate that decreased bone strength occurs in the femoral neck of ovx rats. However, the structural and histological changes underlying the observed biomechanical deterioration of the femoral neck have not been well characterized. Some investigators have addressed this issue. Yamamoto et al.[32] reported that estrogen depletion induces cancellous, but not cortical, bone loss in the femoral neck of ovx rats at relatively early times postovariectomy. We recently found that both cancellous and cortical osteopenia associated with high bone turnover were evident in the femoral neck of ovx rats at 1 year postovariectomy.[10] Despite these findings, the time course of the onset of cancellous and cortical bone loss in the femoral neck of ovx rats as well as the progression to more marked osteopenia remains unclear. This information is critical for the proper design of preclinical studies of the efficacy of therapeutic agents for the prevention or reversal of bone loss in the estrogen-depleted skeleton. Therefore, the objective of the current study was to characterize by histomorphometrical techniques changes in bone mass and turnover in the femoral neck of ovx rats as a function of time from 0 to 360 days postovariectomy.

Materials and Methods

Ninety-day-old female Sprague–Dawley rats (Charles River Laboratory, Inc., Wilmington, MA) weighing an average of 240 g were randomized into 13 groups at the beginning of the study. On the day of surgery (day 0), all rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine hydrochloride and xylazine at doses of 50 and 10 mg/kg body weight, respectively. Eight rats were killed on day 0 as baseline controls (BSL). The remaining rats were subjected to either bilateral ovariectomies (ovx) or sham surgeries (CON). All rats were housed individually at 25°C with a 13 h/11 h light–dark cycle. Food (Teklad 22/5 Rodent Diet, Madison, WI) with Ca and PO_4 contents of 0.95% and 0.67%, respectively, was available ad libitum to the sham-operated control rats. The food consumption of ovx rats was restricted to that of control rats to minimize the increase in body weight associated with ovariectomy.[26] All rats were injected i.p.

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with demeclocycline and calcein (Sigma Chemical Co., St. Louis, MO) on days 10 and 3 before death, respectively, at a dose of 15 mg/kg body weight. This regimen resulted in deposition of a double fluorochrome label at bone surfaces that were actively mineralizing at the times of both injections.

Control and ovx rats were killed by exsanguination from the abdominal aorta under ketamine–xylazine anesthesia at the following times postsurgery: 30, 60, 90, 180, 270, and 360 days. The sample size consisted of eight to ten rats for each of the control and ovx groups at each time point, with the exception of five rats in the control groups at both 180 and 270 days. Success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns. The right proximal femur was stripped of musculature, cut cross-sectionally at 0.5 cm distal to the lesser trochanter with a handheld saw (Dremel Moto Tool, Racine, WI), and placed in 10% phosphate-buffered formalin for 24 h, for tissue fixation.

The lesser trochanter of each sample was scraped with a razor blade on the flexor side to ensure that this surface was flat. The bone samples were dehydrated in ethanol and embedded undecalcified in methyl methacrylate[2] with the flexor side facing down. Before sectioning, each methyl methacrylate block with bone sample was ground carefully with a dental model trimmer (Buffalo Dental Mfg., Syosset, NY) on the flexor side, parallel to the long axis of the femoral neck, until the whole marrow cavity of the proximal femur, including that of the femoral neck, was exposed to ensure uniform and consistent positioning. Bone sectioning was then started at approximately one third the depth of the femoral neck. These longitudinal sections of the proximal femur were cut with an AO Autocut/Jung 1150 microtome at 4 and 8 μm thickness. The sections with the widest marrow cavity near the central part of the femoral neck were selected for further histological processing and histomorphometric measurements. The 4-μm-thick sections were stained according to the Von Kossa method with a tetrachrome counterstain (Polysciences, Warrington, PA) for measurements of cancellous bone volume, femoral neck width, cortical width, osteoblast surface, and osteoclast surface. The 8-μm-thick sections remained unstained for measurements of fluorochrome-based variables.

All bone measurements were performed with the Bioquant Bone Morphometry System (R & M Biometrics Corp., Nashville, TN) as previously described.[30] Cancellous bone measurements were performed in the proximal femur in an area beginning 1 mm distal to the growth plate–metaphyseal junction and extending further distally to the junction of the femoral neck and greater trochanter. The sample site is depicted in **Figure 1.** Cancellous bone volume as a percentage of bone tissue area and osteoblast and osteoclast surfaces as percentages of total cancellous perimeter were measured at a magnification of ×200. Trabecular number, width, and separation were calculated.[10] Femoral neck width and cortical width were measured in one section from each rat at a magnification of ×20. Neck width was defined as the mean distance between opposing periosteal surfaces perpendicular to the long axis of the femoral neck. This distance was measured at three separate sites (approximately 1.5, 2.5, and 3.5 mm distal to the growth plate) and averaged to obtain a mean femoral neck width for each animal. Cortical width was measured from the periosteal to the endocortical surfaces at two separate sites distal to the growth plate at the medial and lateral sides of each femoral neck (Figure 1). The four measurements were averaged to obtain a mean cortical width for each animal.

Fluorochrome-based indices of bone formation, including percentages of cancellous, endocortical, and periosteal surfaces with double fluorochrome labels (mineralizing surface) and mineral apposition rate, were measured with the Bioquant system.

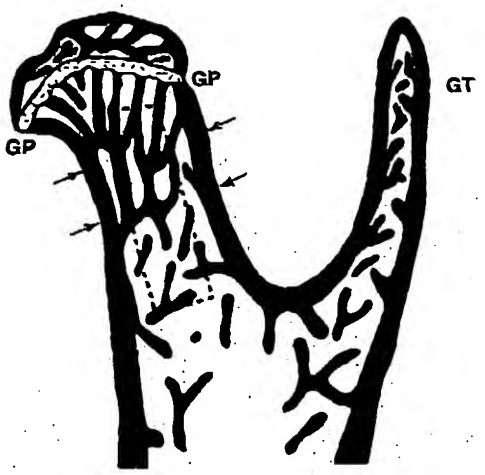

Figure 1. Schematic representation of the proximal femur including the femoral head and neck with growth plate (GP) to the left and the greater trochanter (GT) to the right. The sample area within the femoral neck for measurement of cancellous bone variables is depicted by the broken lines. It begins 1 mm distal to the growth plate and extends nearly to the junction of the femoral neck and greater trochanter. Arrows denote the sites for measurements of cortical width between the periosteal and endocortical surfaces. These sites are approximately 1.5 and 3 mm distal to the growth plate. (Originally published by Li and Wronski in *Bone* 16:552, 1995.)

Bone formation rate (tissue level, surface referent) was calculated by multiplying mineralizing surface by mineral apposition rate.[6] Values for mineral apposition rate were not corrected for obliquity of the plane of section in cancellous bone.[6] In addition, longitudinal bone growth rate was determined by measuring the distance between the fluorescent calcein band that parallels the growth plate and the growth plate–metaphyseal junction at five equally spaced sites per section. This distance was divided by the time interval between administration of the calcein label and death (3 days) to calculate the rate of longitudinal bone growth.

Data are expressed as the mean ± standard error (SE) for the control and ovx groups at each time point. Statistical differences between groups were evaluated with one-way analysis of variance followed by Fisher PLSD.[15] p values < 0.05 were considered to be significant. Within each group, changes in bone histomorphometrical variables as a function of time were evaluated by linear regression analysis.[33]

Results

All rats gained weight during the course of the study. Despite pair-feeding, ovx rats weighed 5%–13% more than control rats. For example, control and ovx rats weighed 448 ± 20 and 506 ± 19 g ($p < 0.05$), respectively, at 1 year postsurgery.

Cancellous bone volume in the femoral neck of control and ovx rats is plotted as a function of time postsurgery in **Figure 2.** Cancellous bone volume (Figure 2A) remained relatively constant at 47%–55% in control rats aged 3–12 months. A slight but significant increase in cancellous bone volume was observed in control rats aged 15 months compared with baseline rats. ovx rats exhibited a significantly lower cancellous bone volume relative to control rats at 30 days postsurgery. Cancellous bone volume in ovx rats gradually declined with time, decreasing to nearly 50% of the mean value for control rats by 180 days postovari-

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ectomy. ovx rats remained osteopenic at the femoral neck out to 360 days postovariectomy. Regression analysis (Table 1) over the entire time course of the study (0–360 days) revealed that cancellous bone volume of the femoral neck remained constant in control rats but declined with time in ovx rats.

Structural data for cancellous bone in the femoral neck are listed in Table 2. In control rats, trabecular number and separation remained relatively constant throughout the study, whereas trabecular width increased with age (Tables 1 and 2). In contrast, ovx rats showed a significant decrease in trabecular number and increase in trabecular separation relative to control rats in a time-dependent manner (Tables 1 and 2), with statistical significance

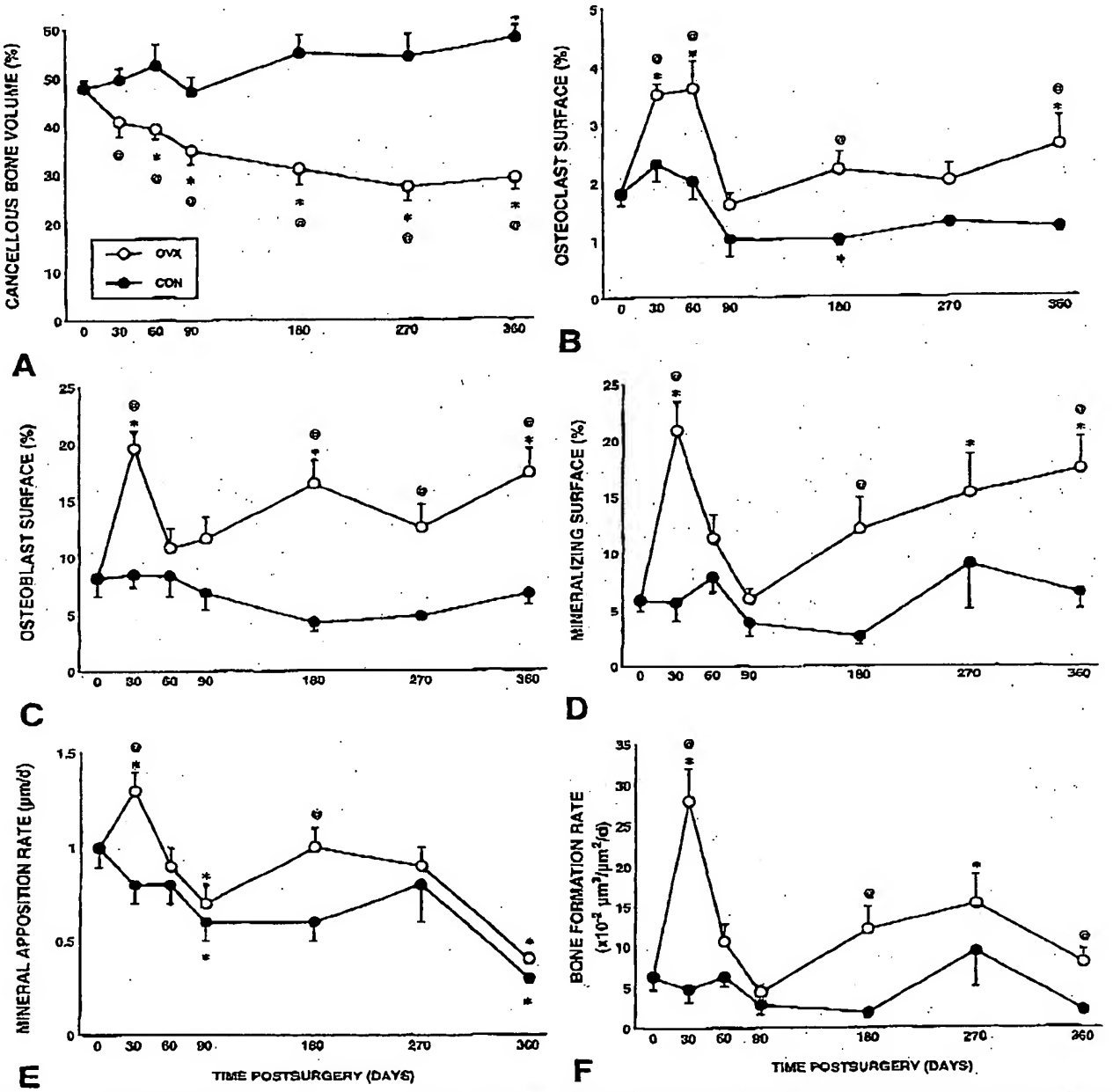

Figure 2. Cancellous bone volume (A), osteoclast surface (B), osteoblast surface (C), mineralizing surface (D), mineral apposition rate (E), and bone formation rate (F) in the femoral neck plotted as a function of time postsurgery. Each data point for the control (closed circle) and ovx (open circle) groups is the mean ± SE of eight to ten animals, with the exception of the control groups at 180 and 270 days (n = 5). The data point for the baseline control group (day 0) is the mean ± SE of eight animals. *Significantly different from baseline control group ($p < 0.05$). °Significantly different from control group at same time point ($p < 0.05$).

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Table 1. Results of linear regression analysis

Variables[a]	Age as independent parameter			ovx as independent parameter		
	Slope	r	p Value	Slope	r	p Value
Cn.BV/TV	1.514	0.344	NS	−3.254	0.644	0.0001
Oc.S/BS	−0.166	0.441	0.0008	−0.064	0.105	NS
Ob.S/BS	−0.357	0.192	NS	0.944	0.273	NS
MS/BS	0.107	0.05	NS	0.892	0.209	NS
MAR	−0.087	0.567	0.0001	−0.083	0.486	0.0001
BFR/BS	−0.414	0.183	NS	−0.688	0.135	NS
Tb.N	−0.088	0.367	NS	−0.406	0.809	0.0001
Tb.Wi	5.684	0.5	0.0001	2.784	0.237	NS
Tb.Sp	−1.49	0.117	NS	35.703	0.723	0.0001
FN.Wi	0.06	0.646	0.0001	0.038	0.471	0.0001
Ct.Wi	0.008	0.301	NS	−0.004	0.132	NS
E.BFR/BS	−12.433	0.729	0.0001	−14.508	0.715	0.0001
P.BFR/BS	−7.212	0.585	0.0001	−9.632	0.585	0.0001

[a]Bone histomorphometric variables from the femoral neck are abbreviated as follows: cancellous bone volume = Cn.BV/TV; osteoclast surface = Oc.S/BS; osteoblast surface = Ob.S/BS; mineralizing surface = MS/BS; mineral apposition rate = MAR; bone formation rate = BFR/BS; trabecular number = Tb.N; trabecular width = Tb.Wi; trabecular separation = Tb.Sp; femoral neck width = FN.Wi; cortical width = Ct.Wi; endocortical bone formation rate = E.BFR/BS; periosteal bone formation rate = P.BFR/BS.

first detected at 60 days postovariectomy. Trabecular width decreased significantly in ovx rats compared with control rats at 180 and 270 days postsurgery.

Data for cell and dynamic histomorphometry of cancellous bone are shown in Figure 2. Osteoclast surface and mineral apposition rate of control rats declined with age (Figures 2B, E, and Table 1). However, osteoblast surface (Figure 2C), mineralizing surface (Figure 2D), and bone formation rate (Figure 2F) did not change significantly in control rats during the course of the study. ovx rats exhibited a maximal increase in osteoclast surface at 30 and 60 days, which then decreased toward the control level at 90 days postsurgery. The values for this variable remained increased in ovx rats relative to control rats at later times and achieved statistical significance at 180 and 360 days postovariectomy. Similarly, osteoblast surface, mineralizing surface, and bone formation rate were maximally increased in ovx rats relative to control rats at 30 days, declined toward control levels at 60–90 days, but remained significantly increased compared with control rats at most of the later times postovariectomy. In addition, ovx rats had significantly increased mineral apposition rate compared with control rats at 30 and 180 days postsurgery.

Longitudinal bone growth rate in the proximal femur of control rats aged 3 months was 5.4 ± 0.3 μm/day and declined to negligible levels < 1 μm/day at 6 months of age. This variable was increased in ovx rats relative to control rats only at 30 days (3.5 ± 0.6 μm/day vs. 2.0 ± 0.2 μm/day; $p < 0.05$), then returned to control level at later times postsurgery (data not shown). Although the growth plate of the proximal femur in both control and ovx rats remained open at the end of the study when these animals were 15 months of age, the fluorescent calcein band that parallels the growth plate was not consistently observed in rats aged 9–15 months. Therefore, the rate of longitudinal bone growth in the femoral neck was minimal and could not be measured in these aged control and ovx rats.

Histomorphometric variables in cortical bone of the femoral neck are listed in **Table 3.** In control rats, femoral neck width

Table 2. Structural variables of cancellous bone in femoral neck[a]

Day[b]	Group[c]	Tb.N[d] (#/mm)	Tb.Wi (μm)	Tb.Sp (μm)
0	BSL	4.8 ± 0.1	100 ± 3	110 ± 4
30	CON	4.8 ± 0.2	105 ± 5	106 ± 8
	ovx	4.5 ± 0.1	92 ± 8	131 ± 8*
60	CON	4.5 ± 0.2	118 ± 9	109 ± 13
	ovx	3.8 ± 0.1*†	105 ± 6	163 ± 8*†
90	CON	4.4 ± 0.2	108 ± 8	125 ± 11
	ovx	3.3 ± 0.3*†	108 ± 11	213 ± 29*†
180	CON	4.4 ± 0.1	126 ± 10*	104 ± 9
	ovx	3.4 ± 0.3*†	93 ± 8†	222 ± 25*†
270	CON	4.0 ± 0.2	135 ± 11*	116 ± 16
	ovx	2.5 ± 0.2*†	106 ± 6†	307 ± 37*†
360	CON	4.4 ± 0.1	133 ± 8*	96 ± 5
	ovx	2.5 ± 0.2*†	120 ± 9	307 ± 32*

[a]Data are expressed as mean ± SE of 8–10 rats/group, with the exception of the control groups at 180 and 270 days ($n = 5$).
[b]Time postsurgery (days).
[c]BSL: baseline control rats; CON: sham-operated control rats; ovx: ovariectomized rats.
[d]Bone histomorphometric variables from the femoral neck are abbreviated as follows: trabecular number = Tb.N; trabecular width = Tb.Wi; trabecular separation = Tb.Sp.
*$p < 0.05$ vs. BSL rats; †$p < 0.05$ vs. CON rats at same time point.

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increased with age (Tables 1 and 3), whereas cortical width did not change significantly between 3 and 15 months of age. No significant difference in femoral neck width was found between control and ovx rats at all time points. However, in comparison to control rats, a slight decrease in cortical width of the femoral neck was observed in ovx rats at 180 and 270 days, but reached statistical significance only at 360 days postsurgery. Endocortical and periosteal bone formation rates decreased significantly with time in control and ovx rats (Tables 1 and 3). ovx rats had higher values than control rats for endocortical bone formation rate at most times postsurgery. A strong trend for increased periosteal bone formation rate was observed in ovx rats relative to control rats at early times postovariectomy (30 and 60 days), but this trend was not statistically significant.

Discussion

The current study indicates that cancellous osteopenia is associated with increased bone turnover occurs in the femoral neck of ovx rats, as has been observed in the proximal tibial metaphyses and lumbar vertebral bodies of these estrogen-depleted animals.[27,28] However, the time course of the osteopenic changes differs at the three skeletal sites. For example, cancellous bone loss in the proximal tibia of ovx rats is statistically significant as early as 14 days postovariectomy. In addition, ovx rats lose ~50% of their cancellous bone mass in the proximal tibial metaphyses by 30–60 days postovariectomy. Equivalent loss of cancellous bone in the femoral neck and lumbar vertebral body does not occur until 180 and 270 days postovariectomy, respectively. These findings indicate that cancellous bone loss in the femoral neck and lumbar vertebra of ovx rats is slower and less pronounced than in the proximal tibia. The observed decrease in cancellous bone mass in the femoral neck of ovx rats was accompanied by a poor architecture and a discontinuous trabecular network, as evidenced by decreased trabecular number and width as well as increased trabecular separation in ovx rats relative to control rats. This skeletal deterioration induced by estrogen depletion in the femoral neck of ovx rats is similar to that observed in osteoporotic patients with hip fractures.[16]

Increases in various histomorphometric indices of bone formation and resorption were observed in the femoral neck of ovx rats. Therefore, cancellous bone loss induced by estrogen depletion at this skeletal site is associated with high bone turnover in which the increment in bone resorption is apparently greater than the increment in bone formation. This finding is consistent with the skeletal effects of estrogen depletion in postmenopausal and oophorectomized women.[7,24] Similar to previous findings in the proximal tibial metaphysis and lumbar vertebral body of ovx rats,[27,28] bone turnover was maximally increased in the femoral neck of these animals at an early time (30 days), declined toward the control level between 60 and 90 days, but then remained increased at later times postovariectomy. However, as mentioned earlier, the rate and magnitude of cancellous bone loss differ at these skeletal sites. The explanation for this difference in the skeletal response to estrogen depletion is unclear. Variations in the baseline rate of bone turnover, rate of bone growth, and mechanical loading may be involved in the observed intraskeletal variation in bone loss in ovx rats.

Cortical bone loss occurred in the femoral neck of ovx rats, as indicated by a thinning of the cortex without a change in neck width. Coincident with this thin cortex, bone formation rate was significantly increased at the endocortical surface of ovx rats throughout the duration of the study. Importantly, eroded perimeter, an index of bone resorption, has been found to be increased at the endocortical surface of the femoral neck in ovx rats compared with control rats, which resulted in an expansion of the marrow cavity in the former animals.[32] These finding suggest that high bone turnover with resorption greater than formation resulting in a negative bone balance at the endocortical surface is the most likely explanation for the development of cortical osteopenia in the femoral neck of ovx rats. However, although increased indices of bone turnover at the endocortical surface of the femoral neck were found in ovx rats at 1–2 months postovariectomy,[32] a significant decrease in cortical width was not observed until 1 year postsurgery in the current study. This finding suggests that the negative bone balance at the endocortical surface is slight, which results in a slow rate of cortical bone loss in the femoral neck of ovx rats. Consistent with the observation

Table 3. Histomorphometric variables of cortical bone in femoral neck[a]

Day[b]	Group[c]	FN.Wi (mm)[d]	Ct.Wi (mm)	E.BFR/BS ($\times 10^{-2}$ μm^3/μm^2 per day)	P.BFR/BS ($\times 10^{-2}$ μm^3/μm^2 per day)
0	BSL	2.45 ± 0.04	0.61 ± 0.02	90.0 ± 9.7	53.3 ± 7.0
30	CON	2.55 ± 0.06	0.61 ± 0.02	58.1 ± 8.0*	61.1 ± 8.0
	ovx	2.64 ± 0.04*	0.61 ± 0.01	99.3 ± 11.8†	87.3 ± 15.2
60	CON	2.59 ± 0.05	0.67 ± 0.01*	10.3 ± 2.9*	17.5 ± 2.3*
	ovx	2.58 ± 0.05	0.65 ± 0.03	37.0 ± 8.8*†	28.0 ± 4.2*
90	CON	2.66 ± 0.04*	0.65 ± 0.02	2.0 ± 0.7*	8.9 ± 1.1*
	ovx	2.60 ± 0.04*	0.67 ± 0.02*	9.3 ± 1.9*†	8.3 ± 1.7*
180	CON	2.79 ± 0.05*	0.65 ± 0.01	4.6*	8.0 ± 1.4*
	ovx	2.73 ± 0.04*	0.62 ± 0.01	12.2 ± 4.4*	19.6 ± 5.4*
270	CON	2.79 ± 0.05*	0.67 ± 0.03	7.5*	28.4 ± 2.8*
	ovx	2.70 ± 0.05*	0.61 ± 0.02	19.4 ± 8.2*	14.6 ± 6.0*
360	CON	2.80 ± 0.06*	0.66 ± 0.02	5.1 ± 1.6	13.5 ± 4.2*
	ovx	2.71 ± 0.06*	0.59 ± 0.02†	12.7 ± 2.4*†	8.7 ± 2.3*

[a]Data are expressed as mean ± SE of 8–10 rats/group, with the exception of the control groups at 180 and 270 days (n = 5).
[b]Time postsurgery (days).
[c]BSL: baseline control rats; CON: sham-operated control rats; ovx: ovariectomized rats.
[d]Bone histomorphometric variables from the femoral neck are abbreviated as follows: femoral neck width = FN.Wi; cortical width = Ct.Wi; endocortical bone formation rate = E.BFR/BS; periosteal bone formation rate = P.BFR/BS.
[e]Value was obtained from one rat owing to the absence of double labels at the endocortical surface in all other rats from this group.
*$p < 0.05$ vs. BSL rats; †$p < 0.05$ vs. CON rats at same time point.

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by Yamamoto et al.,[32] increased periosteal bone formation rate was not evident in the femoral neck as it is in the tibial diaphysis of ovx rats.[9,14,25,31] This finding explains the lack of change in femoral neck width in ovx rats relative to control rats.

Decreased breaking strength of the femoral neck has been reported in ovx rats as early as 6 weeks postovariectomy.[18] Based on histomorphometrical findings in previous studies,[10,32] as well as the current one, it appears that cancellous, but not cortical, bone loss and poor trabecular architecture are the major contributing factors to decreased mechanical strength in the femoral neck of ovx rats at relatively early times postovariectomy.[9,17,18,19] Since cortical bone is thought to play a more important role than cancellous bone in bone strength and fracture resistance,[4,12,21] the observed cortical thinning must undoubtedly contribute to the lower bone strength in the femoral neck of ovx rats at later times postovariectomy.

Age-related cancellous and cortical bone loss was not found in the femoral neck of control rats aged 3–15 months in the current study, which is in agreement with findings by Søgaard et al.[22] However, the possibility that osteopenia eventually develops in more aged control rats cannot be ruled out. Nevertheless, the pattern of age-related changes in cellular and fluorochrome-based bone formation variables detected in the femoral neck of control rats in the current study are similar to those found in the proximal tibia and lumbar vertebra of comparably aged control rats.[27,28] For example, osteoblast surface and mineralizing surface remain relatively constant in control rats aged 3–15 months. However, mineral apposition rate decreased with age at all these bone sites, which is consistent with observations in normal postmenopausal women.[20] These findings indicate that impaired osteoblast activity plays a more important role than altered osteoblast recruitment in age-related bone changes in rats and humans.

Interpretation of histomorphometrical data from long bone metaphyses of growing rats may be complicated by continued bone elongation, especially when a test agent affects longitudinal bone growth. Interestingly, the rate of longitudinal bone growth in the proximal femur of female rats aged 3 months was only 5 μm/day, which is about ten times lower than that of the proximal tibia in female rats of the same age.[11,27] Furthermore, longitudinal bone growth was minimal at <1 μm/day when these rats were 6 months of age. Therefore, the slowly growing proximal femur probably serves as a better sample site than the proximal tibia in the appendicular skeleton of young rats for testing the effects of various agents on cancellous bone by histomorphometrical methods.

In summary, both cancellous and cortical osteopenia associated with high bone turnover occur in the femoral neck of ovx rats. Cancellous bone loss occurs at this skeletal site as early as 30 days postovariectomy, but is relatively moderate for the first 90 days before becoming more pronounced at later times postovariectomy. Cortical osteopenia in the femoral neck is not observed in ovx rats until 1 year postovariectomy. This histomorphometrical characterization of osteopenic changes in the femoral neck of ovx rats may serve as a basis for use of this important sample site in preclinical studies of the prevention and treatment of bone loss in the estrogen-depleted skeleton.

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